AD_____

Award Number: W81XWH-12-1-0213

TITLE: Targeting G-Protein Signaling for the Therapeutics of Prostate Tumor Bone Metastases and the Associated Chronic Bone Pain

PRINCIPAL INVESTIGATOR: Songhai Chen, M.D., PhD.

CONTRACTING ORGANIZATION: University of Iowa
Iowa City, IA 52242

REPORT DATE: July 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2013	Annual	1 July 2012-30 June 2013

4. TITLE AND SUBTITLE
Targeting G-Protein Signaling for the Therapeutics of Prostate Tumor Bone Metastases and the Associated Chronic Bone Pain

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0213

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Songhai Chen (PI)
Yuanchao Ye (Postdoctoral fellow)

E-Mail: Songhai-chen@uiowa.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Iowa, Iowa City, IA 52242

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Bone tumor metastasis is the major cause of mortality and morbidity in advanced prostate cancer patients. These patients frequently suffer from moderate to severe chronic bone pain. However, the current treatments for these patients are ineffective and non-curative, because metastatic tumors are resistant to most of the current anti-cancer treatments, and the currently used pain therapeutic medications or analgesics often do not provide effective relief from pain due to the development of tolerance upon chronic use, and also have serious side effects. Thus, it is imperative to develop novel approaches that can both effectively block tumor growth in bones and relieve the associated bone cancer pain. This proposal aims to define the key role of the Gβγ subunits of heterotrimeric G proteins in the development of prostate tumor bone metastasis and the associated bone pain. Our studies by far have demonstrated that Gβγ signaling plays a key role in mediating proliferation of several prostate cancer cell lines, including LNCaP, PC3, DU145 and 22Rv1. Blocking Gβγ signaling decreases prostate cancer cell growth by increasing apoptosis. Moreover, we show that Gβγ mediates the effect of multiple G protein-coupled receptor-stimulated tumor cell migration and invasion. Finally, we have identified several signaling pathways, including PI3K/AKT, ERK and calcium signaling that are activated downstream of Gβγ and could potential contribute to prostate tumor progression.

15. SUBJECT TERMS
Cancer Pain, Prostate Cancer Bone Metastasis, heterotrimeric G protein βγ subunits, G protein-coupled receptors, signal tranduction

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. Introduction:

Bone metastasis is one of the most common and severe complications in advanced prostate cancer. It is the major cause of mortality and morbidities, due to the development of bone pain, hypercalcemia, fractures, spinal cord compression and consequent paralysis. The current regimen for these patients is largely palliative and non-curative, because metastatic tumors are resistant to most of the current anti-cancer treatments. Thus, it is imperative to develop novel therapeutic approaches for the treatment of advance prostate cancer. This proposal aims to define the key role of the Gβγ subunits of heterotrimeric G proteins in the development of prostate tumor bone metastasis and the associated bone pain, as well as determine the potential therapeutic efficacy of targeting Gβγ with small molecule inhibitors in preclinical models of bone-metastasized prostate cancer. G proteins mediate the function of a large group of cell surface receptor proteins called G protein-coupled receptors (GPCRs). Comparative experimental and clinical evidence has indicated that excessive activation of the GPCR systems due to overexpression of the receptors and their ligands in prostate tumor cells or their surrounding cells contributes to the metastatic spread of tumor cells to bones, their subsequent growth there and the consequent bone destruction. Moreover, continue activation of GPCRs in the sensory nerve fibers adjacent to bones results in increased activity/expression of key pain-sensing receptor channels, such as TRPV1, such that the channels are constitutively activated, leading to the sensation of chronic pain without any overt stimulation. Thus, the GPCR system represents an attractive target for the therapeutics of bone tumor metastasis and the associated bone pain. However, the involvement of several dozen GPCRs and their ligands in tumor progress has presented a significant hurdle for the progression of such approach. Given that G proteins function downstream of GPCRs, we propose to thoroughly investigate the role of Gβγ in mediating signals from multiple GPCRs to promote prostate tumor growth and metastasis and for the associated bone cancer pain, using both *in vitro* cell culture and *in vivo* preclinical model of prostate tumor metastasis. Considering the recent discovery of a series of small molecule inhibitors of Gβγ that have been successfully used in the treatment of several pathologies in the preclinical mouse models of heart failure, inflammation, opioid receptor-dependent analgesia and morphine-induced antinociceptive tolerance and dependence, without causing overt side effects, results from our proposed studies have the potential to uncover a novel and efficacious approach for the development of new mechanism-based therapies to improve the outcome of advanced prostate cancer patients, including the men in the military services who are suffering from this disease.

2. Keywords: Prostate Cancer Bone Metastasis, Bone Cancer Pain, Heterotrimeric G protein betagamma subunits, G protein coupled receptors (GPCRs), TRPV1, Nociceptor Sensitization

3. Overall project summary: Summarized below are the accomplishments from research work performed in the 1st yr of this project in direct alignment with the Statement of Work (SOW) schedule.

Milestone-1: Determine the role of Gβγ signaling in mediating prostate tumor cell growth, migration and invasion *in vitro*, as well as mediating GPCR-regulated TRPV1 channel function in cultured mouse sensory neurons (Aim 1).

Major Goal/Objective 1: Determine the role of Gβγ signaling in regulating prostate tumor cell growth, migration and invasion (months 1-12).

1a. Generation of stable cell lines overexpressing inducible Gβγ or the Gβγ scavenger (months 1-6).
Accomplishments: We have successfully generated lentiviruses encoding firefly luciferase, Gβ1γ2 and the Gβγ scavenger, Gαt. Several prostate cancer cell lines (LNCaP, PC3, DU145 and 22Rv1) and RWPE-1 have been transduced with these lentiviruses to express firefly luciferase together with Gβ1γ2 or Gαt. Western blotting analysis demonstrated that both Gβ1γ2 and Gαt can be induced to express in these cells by treatment with doxycycline (representative data from PC3 cells are shown in Fig. 1A).

1b. Determine the effects of manipulating Gβγ on tumor cell proliferation and apoptosis (months 3-6).
Accomplishments: To determine if Gβγ signaling is required for prostate tumor cell growth, we examined the effect of induced Gβ1γ2 and Gαt expression. As shown in Fig. 1B, D and E, induced Gαt expression decreased PC3 and 22Rv1 cells but not RWPE-1 cell proliferation. Induced Gβγ expression had no effect on PC3 cell proliferation, suggesting that endogenous Gβγ is sufficient to mediate cell growth (Fig. 1C). Analysis of cell apoptosis by Annexin C-FITC staining followed by flow cytometry show that blocking Gβγ signaling

increased prostate tumor cell (DU145, LNCaP, PC3 and 22Rv1) but not RWPE-1 cell apoptosis (Fig. 1F). Together, our data indicate that Gβγ signaling specifically promotes proliferation of prostate tumor cells but not non-transformed prostate epithelial cells, lively through the inhibition of the cell apoptosis pathway. These results are important as it suggests that targeting Gβγ may selectively block proliferation of tumor cells but not normal cells.

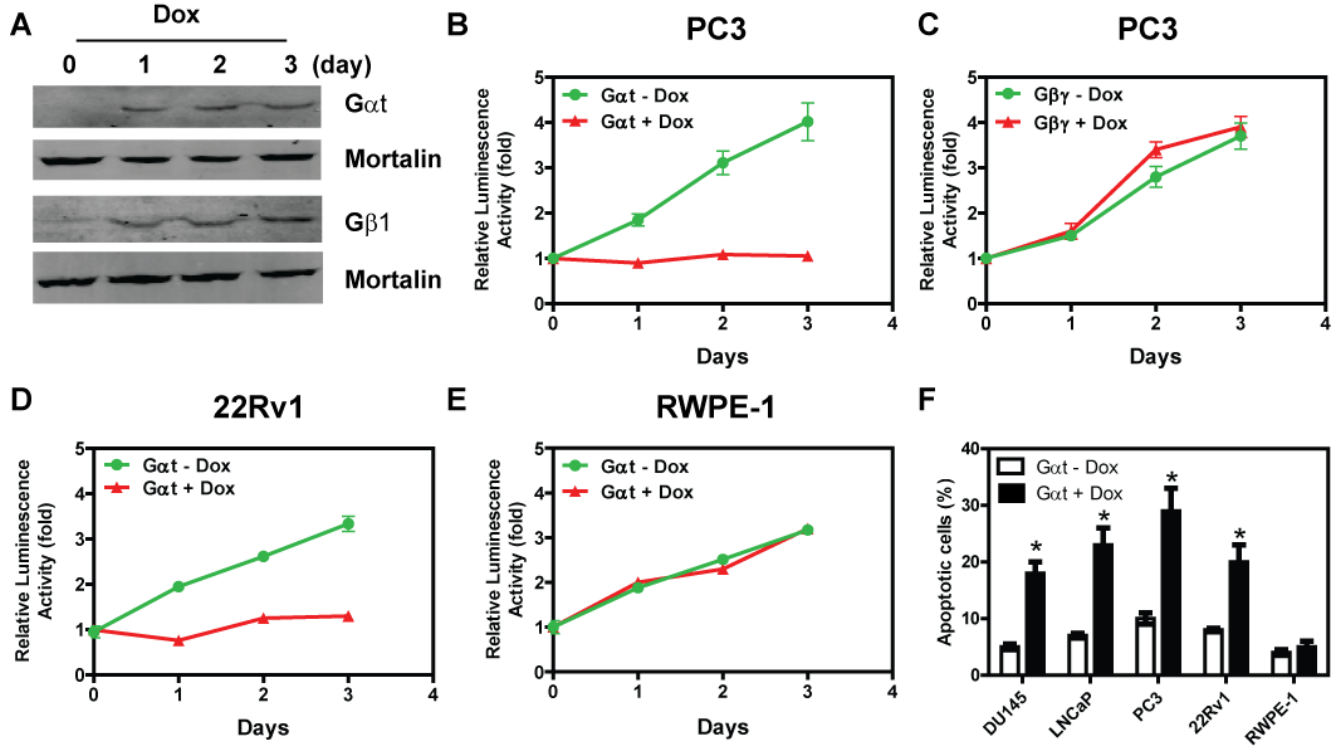

Fig 1. Effects of manipulating Gβγ signaling on prostate tumor cell growth and apoptosis. *A,* representative images showing Gαt and Gβγ expression in PC3 cells induced by doxycycline (Dox, 1μg/ml) treatment. *B-F*, effects of induced Gαt (B, D, E, F) or Gβγ expression (C) on prostate tumor cells or RWPE-1 cell growth (B-E) and apoptosis (F). * p<0.05 indicates significance *versus* Gαt-Dox.

1c. Determine the effects of manipulating Gβγ signaling on tumor cell migration/invasion (months 5-7).
Accomplishments: As shown in Fig. 2A-B, we found that induced Gαt expression blocked several GPCR agonists (including the PAR agonist peptide, IL8, LPA and SDF1α) stimulated PC3 cell migration and invasion by transwell migration assays. Similar findings were observed for other prostate cancer cells (DU145 and 22Rv1) and for invasion (data not shown). These findings thus support our hypothesis that signals from multiple GPCRs converge at Gβγ to promote prostate cancer cell migration and invasion for metastasis formation. These findings are important in light of the fact that tumor metastasis is the major cause of tumor mortality. Currently, we are undertaking experiments to determine the role of Gβγ signaling in mediating prostate tumor metastasis in vivo using a xenograft mouse model.

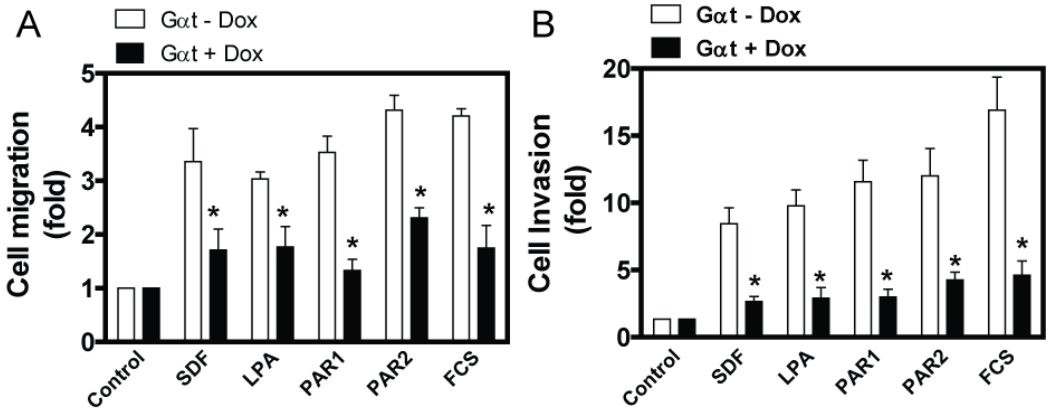

Fig 2. Blocking Gβγ signaling inhibits PC3 cell migration (A) and invasion (B). PC3 cell migration and invasion were induced by the indicated ligands, SDF1α (100nM), LPA (20 nM), PAR1 peptide (10 μM), PAR2 peptide (10 μM) and 1% FCS. * p<0.05 indicates significance *versus* Gαt-Dox.

1d. Determine the effect of manipulating Gβγ signaling on signal transduction (months 6-9).
Accomplishments: Using the stable cell lines we established, we have shown that induced Gαt expression to block Gβγ signaling inhibited protease-activated receptor (PAR) agonist peptide (data not shown) or LPA stimulated Ca^{2+} signaling, AKT and ERK phosphorylation (Fig. 3A-D). These findings indicate that both PAR agonist peptide and LPA transmit signal through Gβγ in prostate cancer cells. Overexpression of Gβ1γ2, however, had no effect on these responses (data not shown), consistent with our observations that the endogenous level of Gβ1γ2 is sufficient to mediate proliferation of prostate cancer cells. Based on these findings, we decided to use prostate cancer cells expressing inducible Gαt to block Gβ1γ2 signaling for further studies.

1e. Determine the role of Gβγ signaling in the transactivation of androgen receptor (months 9-12).
Accomplishments: To determine if Gβγ regulates prostate tumor cell growth and migration in part via regulating androgen receptor activity, we will monitor androgen receptor transcriptional activity in LnCaP and 22v1 with or without Gαt expression. These studies have been delayed because it has taken us a while to obtain luciferase reporter genes for androgen receptor from other investigators. Dr. Eric Bolton (Department of Molecular and Integrative Physiology at the University of Illinois at Urbana-Champaign) are now going to send us two reporter genes for our studies. One of these reporter genes contains the PSA promotor (-4841 to +12) fused to luciferase, while the other contains a 499bp sequence corresponding to ARBR7 with two androgen response elements that are fused to luciferase. We expect to complete this study in 3 months.

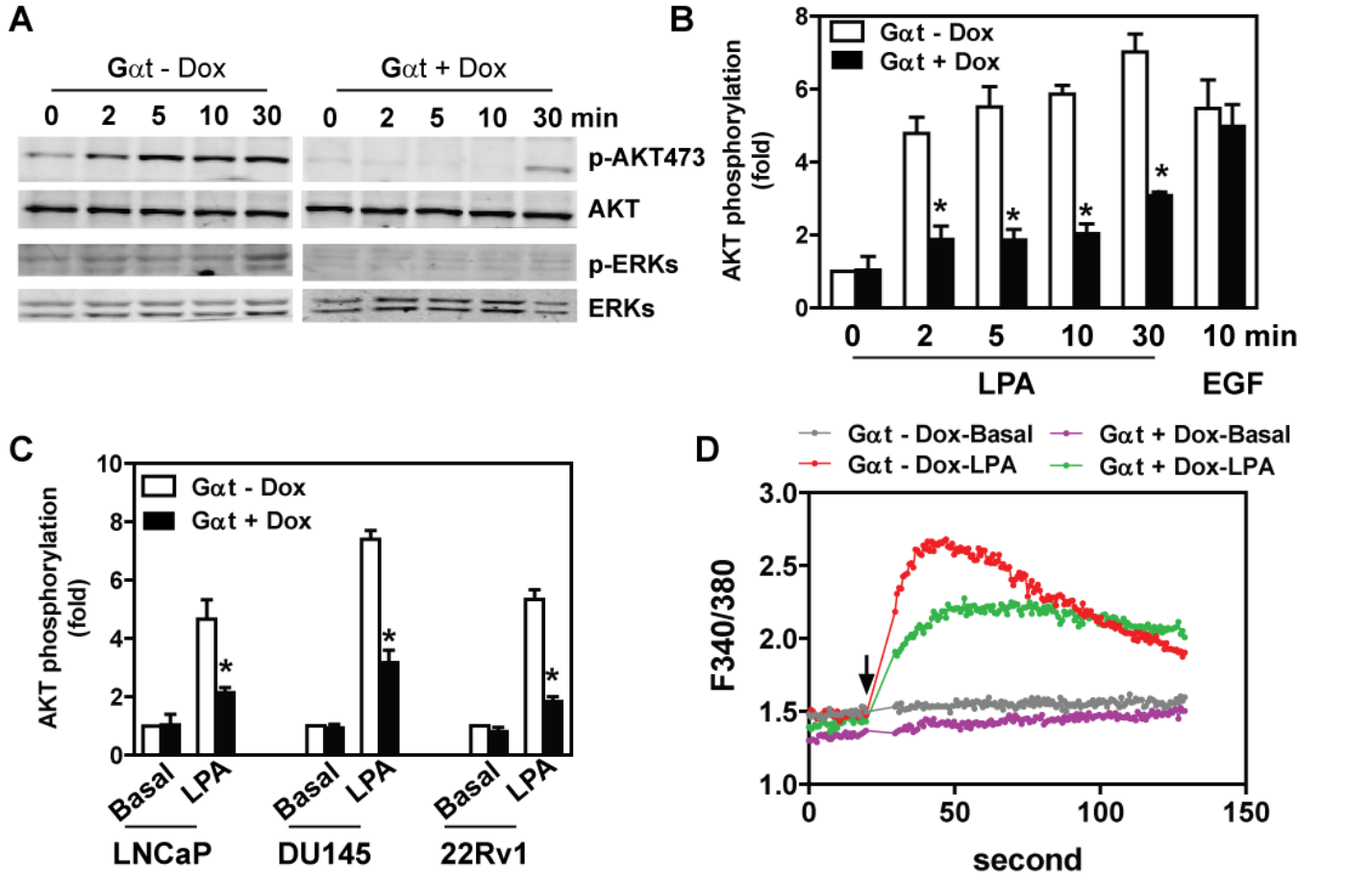

Fig 3. Effects of blocking Gβγ signaling on signal transduction in prostate tumor cells. PC3 (A, B and D) or LNCaP, DU145 and 22Rv1 (C) cells were treated with (Gαt +Dox) or without (Gαt - Dox) to induce Gαt expression. LPA (10 μM) or EGF (20 ng/ml) stimulated AKT and ERK phosphorylation (A-C) and calcium signaling were then measured. *A,* representative images showing LPA-stimulated AKT473 and ERK phosphorylation in PC3 cells; B, quantitative data showing AKT phosphorylation in PC3 cells from three independent experiments; C, quantitative data showing AKT phosphorylation in LNCaP, DU145 and 22Rv1 cells; D, representative data showing calcium signaling induced by LPA in PC3 cells. * $p<0.05$ indicates significance *versus* Gαt-Dox.

Major Goal/Objective 2: Determine the role of Gβγ signaling in mediating GPCR-stimulated upregulation of TRPV1 expression/function in cultured mouse DRG sensory neurons. (months 12-18).

1a. Generating adenovirus encoding EGFP, Gβ1γ2 and Gαt for modulating Gβγ signaling in cultured DRG sensory neurons. (months 12-15)
Accomplishments: We have generated the adenovirus and are currently amplifying the viruses in HEK293 cells for large-scale purification. After the viruses are purified, we will test the efficiency of these viruses to infect cultured DRG. We expect to complete this experiment in two months.

4. Key research accomplishments:

- In our first year of this study, we found that blocking Gβγ signaling inhibits prostate cancer cell growth and migration induced by multiple G protein-coupled receptors. These findings demonstrate that Gβγ serves as a signal convergence point for multiple G protein-coupled receptors that promote prostate cancer progression. Notably, blocking Gβγ signaling does not affect the proliferation of non-transformed prostate epithelial cells. This suggests that it is possible to target Gβγ signaling to specifically block prostate cancer cell growth and migration without affecting normal cell function. These findings thus support our hypothesis that targeting Gβγ signaling may represent a novel therapeutic approach for the treatment of prostate cancer.
- We have generated most of reagents required for validating our findings in vivo in the second year of our studies.

5. Conclusion:

In conclusion, our results from the first year of this study have led us to show that Gβγ signaling mediates the effect of multiple G protein-coupled receptors on promoting prostate tumor cell growth and migration in vitro. Moreover, we have demonstrated that blocking Gβγ signaling inhibits prostate cancer cell growth but has minimal effect on non-transformed prostate epithelial cell growth. These findings thus provide important support for targeting Gβγ signaling as a novel therapeutic approach for prostate cancer. Currently, we are continuing with studies to determine the role of Gβγ signaling in regulating the expression/function of the pain-sensing channel TRPV1. In addition, we will be investigating the function of Gβγ signaling in promoting prostate tumor progression and bone cancer pain in vivo.

6. Publications, Abstracts, and Presentations

Scientific presentations: I was invited to give a talk for the Symposium “New roles for signaling by G-protein betagamma subunits” at the ASPET/EB annual meeting, held in Boston, Massachusetts, April 20-24, 2013. In this symposium, I presented our data on the function of Gβγ signaling in promoting leukocyte migration and progression of tumors including prostate cancer.

7. Inventions, Patents and Licenses: no

8. Reportable Outcomes: n/a

9. Other Achievements: n/a

10. References: n/a

Appendices: n/a